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ANALYSIS OF THE FUNCTIONAL ROLE OF cGMP-DEPENDENT PROTEIN KINASE IN INTACT HUMAN PLATELETS USING A SPECIFIC ACTIVATOR 8-*PARA*-CHLOROPHENYLTHIO-cGMP

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Abstract—8-(*p*-Chlorophenylthio)-cGMP (8-pCPT-cGMP) and 8-bromo-cGMP were compared with respect to their chemical and biological properties in order to evaluate their potential as selective activators of cGMP-dependent protein kinase (cGMP-PK; EC 2.7.1.37) in intact human platelets. 8-pCPT-cGMP, 8-Br-cGMP and cGMP were shown to be potent and selective activators of purified bovine lung cGMP-PK and of cGMP-PK present in human platelet membranes when compared with the activation of cAMP-dependent protein kinase (cAMP-PK; EC 2.7.1.37). 8-pCPT-cGMP was not hydrolysed by the purified cGMP-stimulated phosphodiesterase (cGS-PDE), cGMP-inhibited phosphodiesterase (cGI-PDE) and Ca^{2+}-calmodulin-dependent phosphodiesterase (CaM-PDE), whereas cGMP and, to a lesser extent, 8-Br-cGMP were hydrolysed by all three types of 3′,5′ cyclic nucleotide phosphodiesterases (EC 3.1.4.17) examined. Also, 8-pCPT-cGMP was not hydrolysed by a human platelet homogenate which contains a high level of the cGMP-specific cGMP-binding phosphodiesterase (cGB-PDE). Additionally, 8-pCPT-cGMP did not activate the cGS-PDE or inhibit the cGI-PDE, whereas half-maximal inhibition of cGI-PDE occurred at 8 μM 8-Br-cGMP. The apparent lipophilicity of 8-pCPT-cGMP was higher than that of 8-Br-cGMP. Extracellular application of 8-pCPT-cGMP to intact human platelets reproduced the pattern of protein phosphorylation induced by sodium nitroprusside (SNP), a cGMP-elevating inhibitor of platelet activation. Quantitatively, 8-pCPT-cGMP was more effective than 8-Br-cGMP in inducing phosphorylation of the 46/50 kDa vasodilator-stimulated phosphoprotein, a major substrate of cGMP-PK in intact platelets. As observed with SNP, pretreatment of human platelets with 8-pCPT-cGMP prevented the aggregation induced by thrombin. The results suggest that 8-pCPT-cGMP is a very potent and selective activator of cGMP-PK in cell extracts and in intact human platelets and, in this respect, is superior to 8-Br-cGMP and other cGMP analogs used for intact cell studies. The data also suggest that inhibition of platelet activation in intact human platelets by nitrovasodilators is mediated by cGMP-PK.

Analogs of hormones and other regulatory agents are established tools to elucidate the physiological role and mechanism of action of inter- and intracellular messengers. Cyclic nucleotide derivatives have been used extensively to evaluate the functional role of cyclic nucleotide-dependent protein kinases for the regulation of biological processes [1–5]. However, many tissues and cell types contain more than one cGMP-regulated protein, such as cGMP-dependent protein kinase (cGMP-PK‖), cGMP-stimulated phosphodiesterase (cGS-PDE), cGMP-inhibited phosphodiesterase (cGI-PDE) and cGMP-regulated cation channels [6–9]. Often it is not clear which of these cGMP-regulated proteins mediates the biological effects of cGMP-elevating hormones or drugs.

In human platelets, both cAMP-elevating agents [i.e. prostaglandin I_2 (prostacyclin) and prostaglandin E_1 (PG-E_1)] as well as cGMP-elevating nitrovasodilators [sodium nitroprusside (SNP), endothelium-derived relaxing factor] inhibit activation [8, 10]. Human platelets are known to contain high levels of both cAMP-dependent PK (cAMP-PK) and cGMP-PK [8, 11, 12], a cGI-PDE [7, 13], a cGMP-specific c-GMP-binding phosphodiesterase (cGB-PDE) [14], and even a cGS-PDE [15]. At present, there is no evidence that human platelets contain ion channels directly regulated by cyclic nucleotides. Whereas the cAMP-PK is thought to mediate the inhibitory effects of cAMP on platelet function [8, 10], cGMP-PK [8, 11, 16–18] and/or the cGI-PDE [7, 8, 19, 20] may mediate the inhibitory effects of cGMP on platelet function. Specific cGMP-PK-

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‖ Abbreviations: cAMP-PK, cAMP-dependent protein kinase; cGMP-PK, cGMP-dependent protein kinase; PG-E_1, prostaglandin E_1; VASP, vasodilator-stimulated protein; 8-Br-cGMP, 8-bromo-cGMP; 8-pCPT-cGMP, 8-para-chlorophenylthio-cGMP; Bt_2-cGMP, N^2-2′-O-dibutyryl-cGMP; cGI-PDE, cGMP-inhibited phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase; cGB-PDE, cGMP-binding phosphodiesterase; CaM-PDE, Ca^{2+}/calmodulin-dependent phosphodiesterase; SNP, sodium nitroprusside; SDS, sodium dodecyl sulfate.

mediated protein phosphorylation has been shown to correlate with the inhibitory effects of nitrovasodilators on platelet activation [16–18].

However, nitrovasodilators slightly increase platelet cAMP levels under certain conditions thought to be due to cGMP-mediated inhibition of the cGI-PDE [19, 20] which may also result in platelet inhibition due to activation of cAMP-PK. Therefore, cGMP analogs were evaluated with respect to their potential as selective activators of the cGMP-PK in intact human platelets in order to define the functional role of cGMP-PK. A cGMP-analog useful as a selective activator of the cGMP-PK in intact cells must fulfil the following criteria: (a) high lipophilicity, in order to penetrate the cell membrane and to reach an intracellular concentration sufficient to activate the endogenous cGMP-PK; (b) high specificity for cGMP-PK, required to avoid effects on other regulatory proteins; (c) resistance to hydrolysis by phosphodiesterases to avoid accumulation and possible side effects of analog metabolites; (d) no effect on the catalytic site of phosphodiesterases, required to avoid accumulation of endogenous cyclic nucleotides due to PDE inhibition; and (e) specific and quantitative activation of cGMP-PK mediated protein phosphorylation in intact cells without affecting the cAMP-PK.

In the present study, 8-Br-cGMP, often used as cGMP-analog in intact cell studies, and 8-pCPT-cGMP were compared with respect to the criteria summarized above. These studies were possible since purified cyclic nucleotide-regulated protein kinases and phosphodiesterases have become available [7, 8] and methods were established which permit the analysis of cGMP-PK-mediated phosphorylation in cell extracts and in intact human platelets [16–18]. The results demonstrated that 8-pCPT-cGMP is a useful selective activator of cGMP-PK in intact human platelets.

MATERIALS AND METHODS

Materials. PG-E_1, cAMP, cGMP, 8-bromo-cGMP (8-Br-cGMP) and thrombin were purchased from Sigma (Munich, F.R.G.), and 8-pCPT-cGMP was synthesized according to the methods of Miller *et al.* [21] and Genieser *et al.* [22]. [γ-^{32}P]ATP (3000 Ci/mmol), $^{32}PO_4^{3-}$ and ^{125}I-Protein A (30 mCi/mg) were obtained from Amersham Buchler (Braunschweig, F.R.G.), Dipyridamol (Persantin®) was purchased from Thomae (Biberach, F.R.G.). and Kemptide was obtained from Peninsula (Merseyside, U.K.). All other chemicals were from commercial sources as described previously [11, 16–18].

Protein kinase assay. The type II cAMP-PK and the soluble type I cGMP-PK were purified from bovine heart and bovine lung, respectively, as described earlier [23, 24]. The activity of the purified kinases was measured by the phosphocellulose method [25] using Kemptide as a substrate with minor modifications [26].

Phosphodiesterase assays. Calmodulin-dependent phosphodiesterase (CaM-PDE), cGS-PDE and cGI-PDE were purified using specific monoclonal antibodies [27]. The velocity of hydrolysis (V) was measured by the phosphate release assay [28] using a final concentration of 500 μM cyclic nucleotides. The hydrolysis rates for cAMP were 0.75 nmol/min for cGS-PDE, 0.25 nmol/min for cGI-PDE and 0.3 nmol/min for CaM-PDE. In addition to the cyclic nucleotide analogs reported here, many others were analysed in these phosphodiesterase experiments, and a full account of this work will appear as a separate study (Butt E and Beavo JA, unpublished experiments).

Phosphodiesterase activity of a platelet homogenate. Platelet homogenates were isolated as described previously [11]. The incubation mixture contained 10 μL of the homogenate (210 μg of protein, equivalent to 10^8 platelets), 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.5 mM cGMP and/or 0.5 mM 8-pCPT-cGMP in a final volume of 100 μL. The mixture was incubated at 30° for 15 min and subsequently centrifuged at 6000 g for 2 min. Ten microlitres of the supernatant were directly applied to a reversed phase HPLC (Merck LiChrosorb RP-18 column, 4 × 250 mm, 10 μM) and eluted by a non-linear gradient of acetonitrile/10% triethylammonium formiate buffer. The elution of cyclic nucleotides or metabolites (primarily guanosine-5-'phosphate) was followed by measuring the absorbance at 254 nm. Sample recovery was calculated by measuring the elution of guanosine or 8-pCPT-guanosine which had been added to the sample (final concentration 0.5 mM) immediately before HPLC analysis. Incubations with heat-inactivated homogenates (10 min boiling) were used as control.

Analysis of lipophilicity. Log K_w data of cAMP analogs were determined by reversed phase HPLC as described [29] using a 4 × 250 mm column containing LiChrosorb RP-18, 10 μm material (obtained from Merck, Darmstadt, F.R.G.). Lipophilicity is measured as the partition behaviour between the non-polar bonded stationary phase and the polar eluent [29].

Endogenous phosphorylation of platelet membranes. Membranes of human platelets were isolated and used in phosphorylation experiments as described previously [11] with minor modifications. The incubation mixture (final volume, 140 μL) contained 10 mM Hepes buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 1 mM isobutylmethylxanthine, cyclic nucleotides as indicated, 42 μg of membrane protein and 4 μM [γ-^{32}P]ATP (15,800 cpm/pmol). The phosphorylation reaction was initiated by the addition of ATP, carried out for 2.5 min at 30°, terminated by the addition of a sodium dodecyl sulfate (SDS)-containing stop-solution and boiling, and subsequently analysed by SDS–PAGE and autoradiography (11).

Phosphorylation experiments with intact human platelets. Washed human platelets were prepared as described previously [16–18] and resuspended in an isotonic buffer containing 10 mM Hepes (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose and 1 mM EDTA at a density of 1 × 10^9 cells/mL. For experiments using ^{32}P-labeled platelets, cells were washed once with the isotonic Hepes buffer and subsequently incubated at a density of 5 × 10^9 platelets/mL for 1 hr at 37° in 100 μL of the isotonic

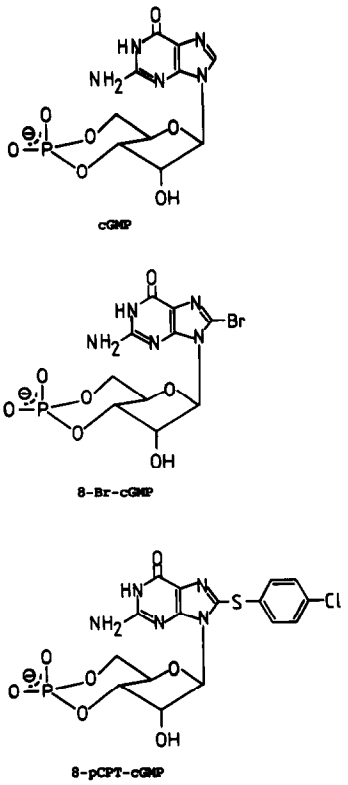

Fig. 1. Structures of cGMP and analogs used.

Table 1. Some properties of cAMP, cGMP and cGMP analogs

Cyclic nucleotides	K_a (μM) cAMP-PK	K_a (μM) cGMP-PK	Lipophilicity (log K_w)
cAMP	0.08	39.0	1.05
cGMP	60.0	0.11	0.68
8-Br-cGMP	12.0	0.01	1.42
8-pCPT-cGMP	7.0	0.04	2.86

The cyclic nucleotides shown were analysed with respect to their half-maximal activation (K_a) of type II cAMP-PK or type I cGMP-PK and with respect to their lipophilicity (log K_w) as measured by HPLC.

Data represent means of at least three separate experiments.

Hepes buffer containing 1 mCi carrier-free $^{32}PO_4^{3-}$. Excess $^{32}PO_4^{3-}$ was removed by washing, and ^{32}P-labeled platelet aliquots were then incubated at 37° with reagents for the times indicated as described previously [16]. Incubations were stopped by the addition of an SDS-containing stop-solution and boiling, and were then analysed by SDS–PAGE and autoradiography [16]. For the quantitative analysis of cGMP-PK-mediated VASP phosphorylation [17, 18], platelet suspensions (final density 1×10^9 cells/mL isotonic Hepes buffer) were incubated at 37° with the reagents indicated. At various incubation times, platelet aliquots were removed, mixed with an SDS-containing stop-solution and boiled. VASP phosphorylation was then measured by SDS–PAGE and western blot analysis as described previously [17, 18].

Platelet aggregation analysis. Human platelets were isolated as described above except that EDTA was omitted and 1 mM $CaCl_2$ and 2 mM $MgCl_2$ were added to the final resuspension buffer. Platelets (final density 1×10^9 cells/mL) were incubated with or without cyclic nucleotides at 37° for 10 min before aggregation was induced by the addition of 0.2 U/mL thrombin. Aggregation was monitored with a platelet aggregation profiler PAP 4 (Bio Data Corporation) measuring light transmission.

RESULTS

The structures of cGMP, 8-Br-cGMP and 8-pCPT-cGMP are shown in Fig. 1. Compared with the natural cyclic nucleotide cGMP, 8-Br-cGMP and 8-pCPT-cGMP are more potent activators of cGMP-PK, whereas they are 100–1000-fold less potent as cAMP-PK activators when compared with cAMP (Fig. 2, Table 1). The cGMP-analogs investigated differ in their apparent lipophilicity. 8-Br-cGMP and 8-pCPT-cGMP are about 6- and 90-fold, respectively, more lipophilic than cGMP (Table 1).

Since human platelets contain several phosphodiesterases which bind and/or degrade cGMP, we investigated the capacity of the cyclic nucleotides to serve as substrates for three different purified cyclic nucleotide phosphodiesterases (Table 2). 8-Br-cGMP was hydrolysed to a small extent by CaM-PDE, cGI-PDE and cGS-PDE, whereas 8-pCPT-cGMP was not hydrolysed detectably by any of the three isozymes of PDE investigated (Table 2). Furthermore, 8-Br-cGMP inhibited the cGI-PDE with a K_i of 8 μM whereas 8-pCPT-cGMP was a poor inhibitor of cGI-PDE (K_i) of 0.38 mM) and did not activate the cGS-PDE (Table 2).

The possible contribution of the cGB-PDE in 8-pCPT-cGMP metabolism in human platelets was also investigated. A human platelet homogenate hydrolysed cGMP to guanosine-5′-phosphate which was partially (>50%) inhibited by 10 μM Dipyridamol (Table 3) indicating the presence and activity of the cGB-PDE in these platelet homogenates [7, 14, 28]. However, these platelet homogenates did not hydrolyse 8-pCPT-cGMP under similar conditions and the cGMP hydrolysis was not significantly affected by the presence of 8-pCPT-cGMP (Table 3).

In agreement with previous studies [11, 30], the activation of cAMP-PK in platelet membranes by cAMP resulted in the phosphorylation of specific substrate proteins with molecular masses of 22, 24, 50, 68, 130 and 240 kDa (Fig. 3). In contrast, cGMP caused cGMP-PK-mediated phosphorylation [11, 30] of three proteins with molecular masses of 46, 50, and 130 kDa (Fig. 3), an effect reproduced by 8-pCPT-cGMP (Fig. 3). Using specific inhibitors of cAMP-PK (protein kinase inhibitor) or of cGMP-PK (antiserum) it has been demonstrated with platelet membranes that the cGMP- and 8-pCPT-cGMP-stimulated phosphorylation of the 46-, 50-,

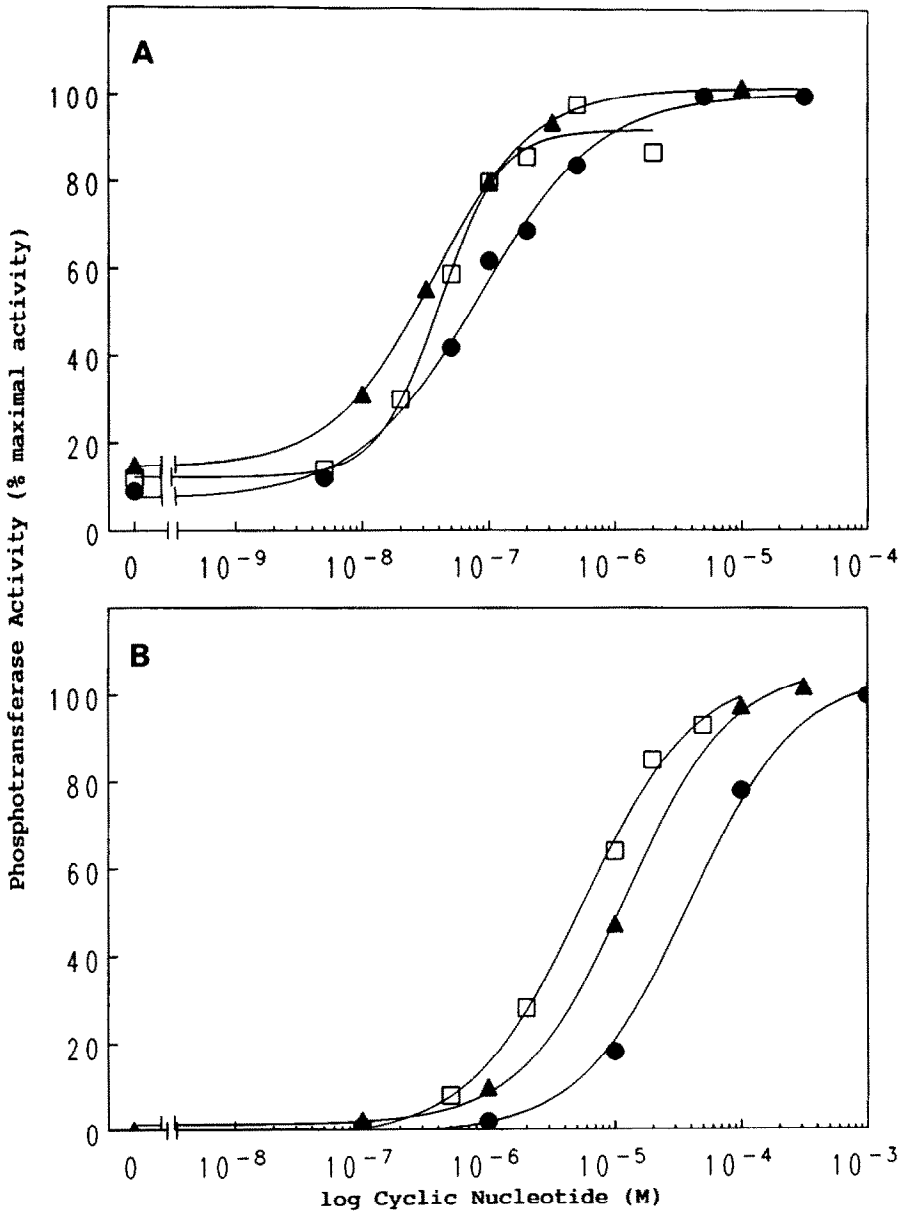

Fig. 2. Effects of cyclic nucleotide analogs on the activity of purified cyclic nucleotide-dependent protein kinases. The concentration-dependent activation of cGMP-PK (A) or cAMP-PK (B) by cGMP (●), 8-Br-cGMP (▲) and 8-pCPT-cGMP (□) is shown. The data represent means of closely agreeing triplicates.

Table 2. Some properties of cyclic nucleotide phosphodiesterases

Cyclic nucleotides	Activation K_a (μM) of cGS-PDE	Inhibition K_i (μM) of cGI-PDE	Hydrolysis (V')		
			cGS-PDE	cGI-PDE	CaM-PDE
cAMP			1.0	1.0	1.0
cGMP	0.35	0.14	1.0	0.3	0.7
8-Br-cGMP	1250	8.0	0.025	0.08	0.06
8-pCPT-cGMP	no act.	385	—	—	—

The cyclic nucleotides shown were analysed with respect to their half-maximal activation K_a of the cGS-PDE or half-maximal inhibition K_i of the cGI-PDE and with respect to their hydrolysis V' by the cGS-PDE, the cGI-PDE and the CaM-PDE.

The hydrolysis data are expressed as the velocity of hydrolysis of the analog relative to cAMP hydrolysis [$V' = V$(analog)/V(cAMP)] at a substrate concentration of 0.5 mM and represent the means of triplicates of two separate experiments. —, No hydrolysis.

Table 3. Phosphodiesterase activity of platelet homogenates

Conditions	Cyclic nucleotide hydrolysis (%)
Control, cGMP	0
Control, 8-pCPT-cGMP	0
cGMP	70–80
cGMP + Dipyridamol	30
8-pCPT-cGMP	0
cGMP + 8-pCPT-cGMP	70 (cGMP) 0 (8-pCPT-cGMP)

A platelet homogenate was incubated for 15 min with 0.5 mM cGMP and/or 0.5 mM 8-pCPT-cGMP and other additions as indicated. The extent of cyclic nucleotide hydrolysis was determined by HPLC. Incubations with heat-inactivated platelet homogenates were used as controls. The results are representative for three experiments using two different platelet preparations.

and 130-kDa proteins is mediated by the cGMP-PK whereas the cAMP-induced phosphorylation of the 22-, 24-, 50-, 68-, 130- and 240-kDa proteins is mediated by the cAMP-PK [11, 27] (data for 8-p-CPT-cGMP not shown). The small extent of the 240-kDa protein phosphorylation by both cGMP and 8-pCPT-cGMP (Fig. 3) is due to a small degree of cAMP-PK activation under these conditions [11, 28] (data not shown).

To investigate whether extracellularly applied 8-pCPT-cGMP is capable of activating the intracellular cGMP-PK, phosphorylation experiments were carried out with intact platelets. The cGMP-elevating nitrovasodilator SNP stimulated the phosphorylation of a 50-kDa protein, an effect also observed with 1.0 mM extracellular 8-pCPT-cGMP (Fig. 4). Maximal phosphorylation of the 50-kDa protein in intact platelets was observed within 10 min of SNP treatment (Fig. 4; compare also with Fig. 5). In contrast, the cAMP-elevating PG-E_1 stimulated the phosphorylation of proteins with molecular masses of 22, 24, 50, 68 and 240 kDa (Fig. 4). This pattern of protein phosphorylation was never seen with 8-pCPT-cGMP or with SNP.

One of the major cGMP-PK substrates in platelet membranes and in intact human platelets is the membrane-associated 46/50 kDa protein termed VASP (the 46/50 kDa phosphoprotein in Figs 3 and 4) which was recently purified and characterized [17, 30]. Phosphorylation of VASP at one site resulted in a shift of its apparent molecular mass in SDS–PAGE from 46 to 50 kDa. This property and the availability of a specific antibody which recognizes both the phospho- (50 kDa) and the dephospho-form (46 kDa) was used to quantitatively measure the cGMP-PK-mediated VASP phosphorylation in intact human platelets [17, 18]. 8-Br-cGMP and 8-pCPT-cGMP caused the time-dependent phosphorylation of VASP indicated by disappearance of the 46-kDa form and appearance of the 50-kDa form (not shown). A comparison of the time-courses and extent of VASP phosphorylation demonstrated that 50% of VASP phosphorylation occurred with 1 mM 8-Br-cGMP only after 60 min, whereas a similar phosphorylation extent was reached with 0.5–1.0 mM 8-pCPT-cGMP after 10 min (Fig. 5). With 0.1 mM SNP, the maximal extent of VASP phosphorylation was reached within 5 min (Fig. 5) which was preceded by a 10-fold evaluation of platelet cGMP levels without significant effects on platelet cAMP levels [17] (data not shown).

The effect of 8-pCPT-cGMP on platelet aggregation was also investigated since cGMP elevating nitrovasodilators such as SNP inhibit the platelet aggregation induced by agonists such as thrombin and collagen. Pretreatment of washed human platelets with 0.5 mM 8-pCPT-cGMP for 10 min prevented the subsequent aggregation in response to thrombin (Fig. 6). Under similar conditions, lower concentrations of 0.1 mM and 0.05 mM 8-pCPT-cGMP caused partial and no inhibition, respectively, of thrombin-induced platelet aggregation (Fig. 6). With 8-Br-cGMP, concentrations of 0.5–1.0 mM were required to obtain complete inhibition of thrombin-induced platelet aggregation [16, 17].

DISCUSSION

One major goal of this study was to evaluate whether cGMP analogs could be used as specific activators of the cGMP-PK with intact cell preparations. A useful cGMP-PK activator should be helpful in distinguishing whether a biological response of cGMP-elevating agents (hormones, drugs etc.) is mediated by the cGMP-PK, the cAMP-PK or by other cGMP-regulated proteins. cGMP-analogs most frequently used with intact cell preparations are N^2-2′-*O*-dibutyryl-cGMP (Bt_2-cGMP), 8-Br-cGMP and more recently 8-pCPT-cGMP. However, Bt_2-cGMP cannot be considered as a useful cGMP-PK activator since it activates the cAMP-PK better than the cGMP-PK, is metabolized to N^2-monobutyryl-cGMP releasing free butyrate (which may have unwanted side effects) and is in general very ineffective when used with intact cell preparations [4]. Furthermore, Bt_2-cGMP and other butyrated cGMP derivatives have been described as antagonists for certain gastrointestinal hormones at the receptor level, an effect unrelated to a specific cGMP binding site [31–34]. In agreement with previous studies [3, 4], both 8-Br-cGMP and 8-pCPT-cGMP were found to be potent and, when

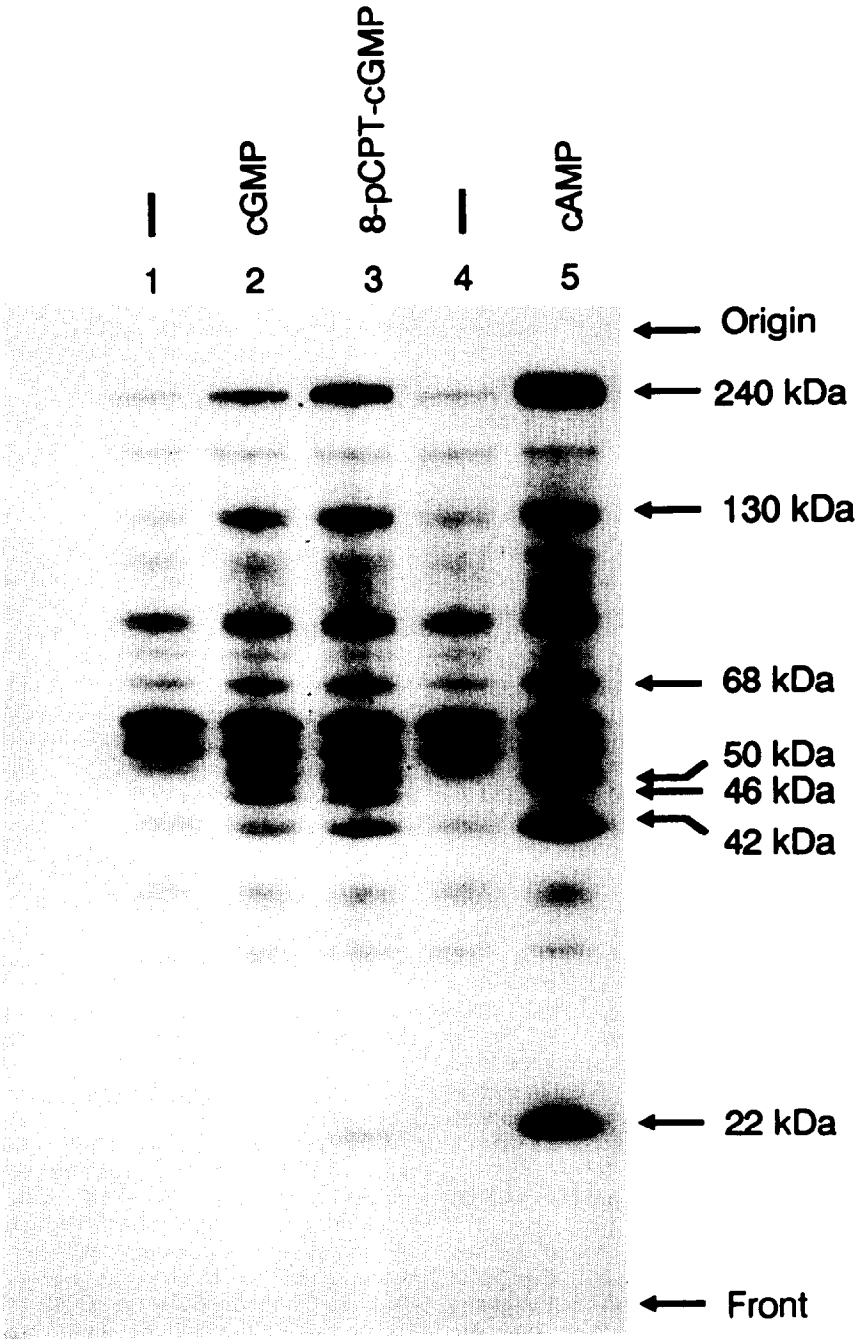

Fig. 3. Autoradiograph showing the effects of cyclic nucleotides on the endogenous phosphorylation in platelet membranes. Phosphorylation of platelet membrane proteins (42 μg) was performed under standard conditions in the absence of cyclic nucleotides (lanes 1 and 4) or in the presence of 1 μM cGMP (lane 2), 1 μM 8-pCPT-cGMP (lane 3), or 1 μM cAMP (lane 5). ^{32}P-Labeled proteins were separated by SDS–PAGE and visualized by autoradiography. Arrows indicate the position and apparent molecular mass of proteins whose phosphorylation was consistently stimulated by these cyclic nucleotides.

compared with the activation of cAMP-PK, selective activators of purified cGMP-PK and cGMP-PK present in human platelet membranes (Figs 2 and 3; Table 1). However, previous studies did not analyse the effects of cGMP analogs with respect to cGMP-regulated phosphodiesterases nor were quantitative data given with respect to the activation of the cGMP-PK in intact cells. 8-Br-cGMP was, although relatively poorly, hydrolysed by three different phosphodiesterases and inhibited the cGI-PDE when used at micromolar concentrations (Table 2). In contrast, 8-pCPT-cGMP was not detectably

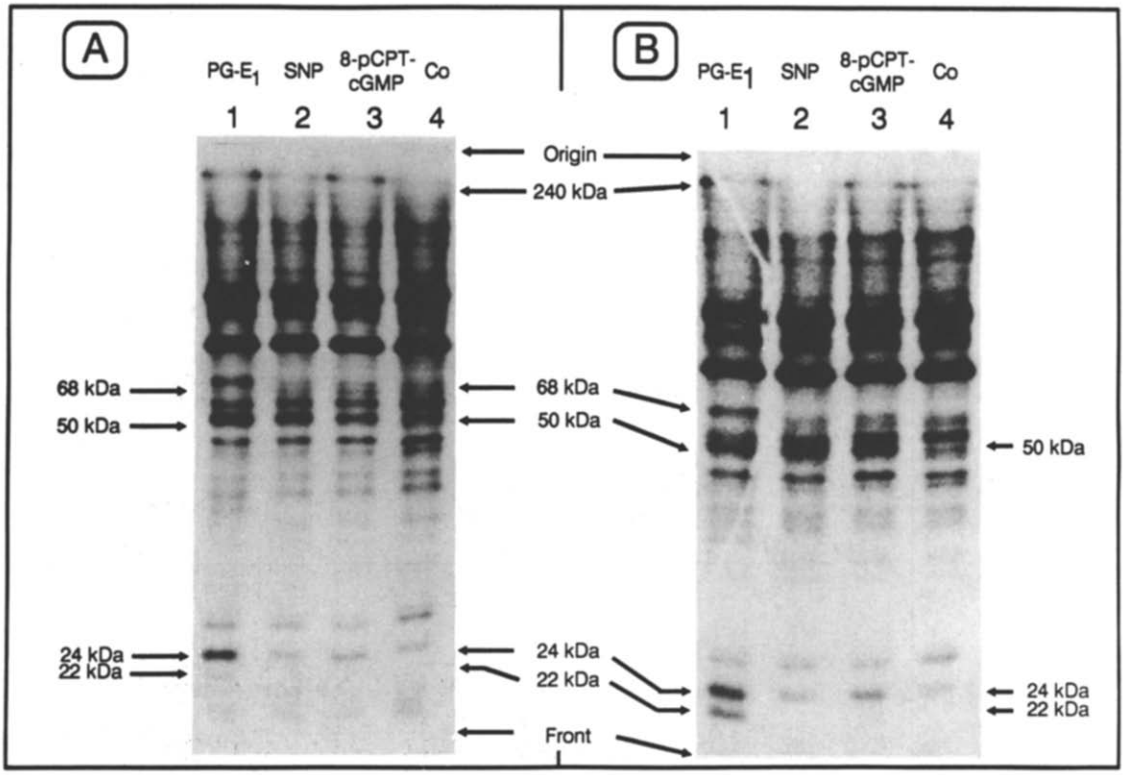

Fig. 4. Autoradiograph showing the regulation of protein phosphorylation in intact platelets by PG-E_1, SNP and 8-pCPT-cGMP. ^{32}P-Labeled platelets were incubated with 5 μM PG-E_1 (lane 1), 0.1 mM SNP (lane 2), 1.0 mM 8-pCPT-cGMP (lane 3) or without additions (lane 4). Incubations were carried out for 10 min (A) or 30 min (B). ^{32}P-Labeled proteins were separated by SDS–PAGE and visualized by autoradiography. The positions and apparent molecular masses of proteins whose phosphorylation was consistently stimulated by the reagents studied are shown.

hydrolysed by the three purified phosphodiesterase examined or by the cGB-PDE of platelet homogenates, and 8-pCPT-cGMP had little if any effect on the activity of cGS-PDE, cGB-PDE or cGI-PDE (Tables 2 and 3). These properties (potent, selective activation of cGMP-PK and no biologically significant effect on cGMP-regulated phosphodiesterase) as well as the high lipophilicity of 8-pCPT-cGMP (Table 1) would predict that this cGMP analog is a good activator of the cGMP-PK in intact cells. This was indeed observed in phosphorylation experiments with intact human platelets, an established model system for the analysis of cGMP-PK-mediated protein phosphorylation [8, 16–18]. Treatment of ^{32}P-labeled platelets with SNP and 8-pCPT-cGMP caused significant phosphorylation of only the 50-kDa protein, termed VASP (Fig. 4), a substrate for both cGMP-PK and cAMP-PK [17, 30]. Increased phosphorylation of substrates for only the cAMP-PK (e.g. the proteins with apparent molecular masses 68, 24 and 22 kDa, Refs 16–18) was observed with the cAMP-elevating PG-E_1 (Fig. 4) and with cAMP analogs [15] but with neither SNP nor 8-pCPT-cGMP (Fig. 4). These results suggest that 8-pCPT-cGMP as well as SNP primarily activated the cGMP-PK and not the cAMP-PK under the conditions used with human platelets, in agreement with the properties of 8-pCPT-cGMP observed with purified enzymes. Since the cGMP-PK-mediated phosphorylation of the 50-kDa protein VASP of intact human platelets can be measured quantitatively using an immunological method [17, 18], it was possible to compare the time-course and extent of cGMP-PK-mediated VASP phosphorylation induced by SNP, 8-Br-cGMP and 8-pCPT-cGMP (Fig. 5). SNP (0.1 mM) converted up to 50% of VASP to the phosphoform within 5 min (Fig. 5), an effect mediated by the cGMP-PK and preceded by a 10-fold increase in platelet cGMP level with no increase in platelet cAMP level [17] (data not shown). Compared to the effect of SNP, VASP phosphorylation induced by 0.5 mM 8-pCPT-cGMP was similar in onset, time-course and maximal extent although VASP phosphorylation increased or declined somewhat after prolonged incubation with 8-pCPT-cGMP or SNP (Fig. 5). In contrast, VASP phosphorylation in response to 8-Br-cGMP had a much slower onset, and the level of 50% VASP phosphorylation was reached only within 30 to 60 min of incubation time (Fig. 5). These phosphorylation data suggest that 8-pCPT-cGMP is considerably more effective than 8-Br-cGMP in penetrating the cell membrane and in reaching an intracellular concentration sufficient to activate

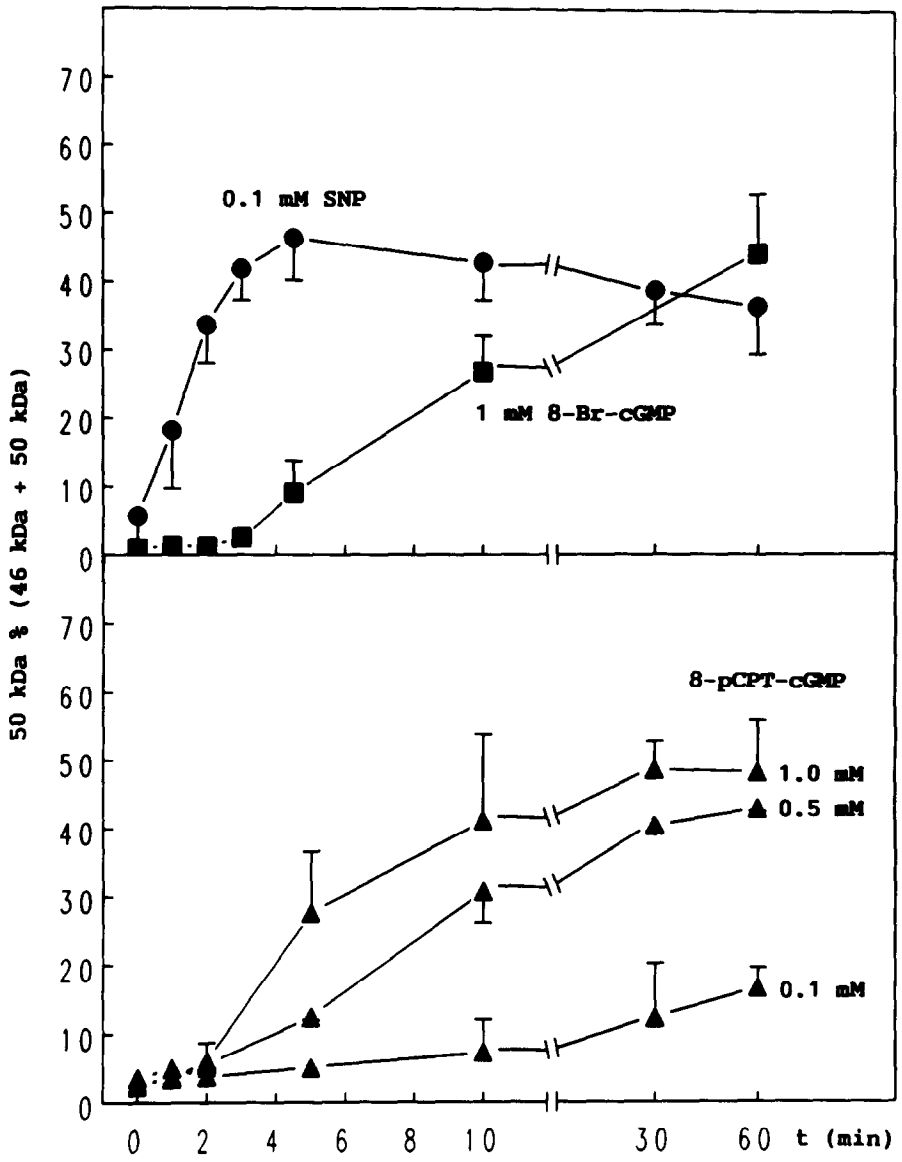

Fig. 5. Quantitative analysis of VASP phosphorylation in platelets incubated with SNP, 8-Br-cGMP or 8-pCPT-cGMP. Intact washed human platelets were incubated with 0.1 mM SNP, 1 mM 8-Br-cGMP (upper panel) and various concentrations of 8-pCPT-cGMP for the times indicated (lower panel). Platelet aliquots were analysed for VASP phosphorylation by the western blot method. VASP phosphorylation is expressed as the percentage phospho-VASP (50-kDa protein) of total VASP (46-kDa + 50-kDa protein). The data represent the means ± SD of three separate experiments.

cGMP-PK, in agreement with the lipophilicity data of these cGMP analogs (Table 1). The higher resistance of 8-pCPT-cGMP to phosphodiesterase hydrolysis may be an additional reason for the higher efficacy of 8-pCPT-cGMP when compared with 8-Br-cGMP. Since human platelets contain cGMP-PK levels of about 4 μM equivalent to 16 μM cGMP binding sites [12, 17], micromolar concentrations of intracellular cGMP (or cGMP analogs) may be necessary to activate significant amounts of the cGMP-PK. Such cGMP (cGMP analog) concentrations appeared to be reached in human platelets within a 5-min incubation with 0.1 mM SNP or 0.5 mM 8-pCPT-cGMP and within 30 to 60 min with 1 mM 8-Br-cGMP.

The aggregation of human platelets was also potently inhibited by 8-pCPT-cGMP (Fig. 6). It is interesting to note that the threshold concentration of 8-pCPT-cGMP required to observe cGMP-PK-mediated VASP phosphorylation and platelet inhibition was about 0.1 mM, similar for both events (Figs 5, 6).

In conclusion, 8-pCPT-cGMP is a more potent and more selective activator of cGMP-PK in intact human platelets than 8-Br-cGMP. Our results obtained with purified protein kinases and phospho-

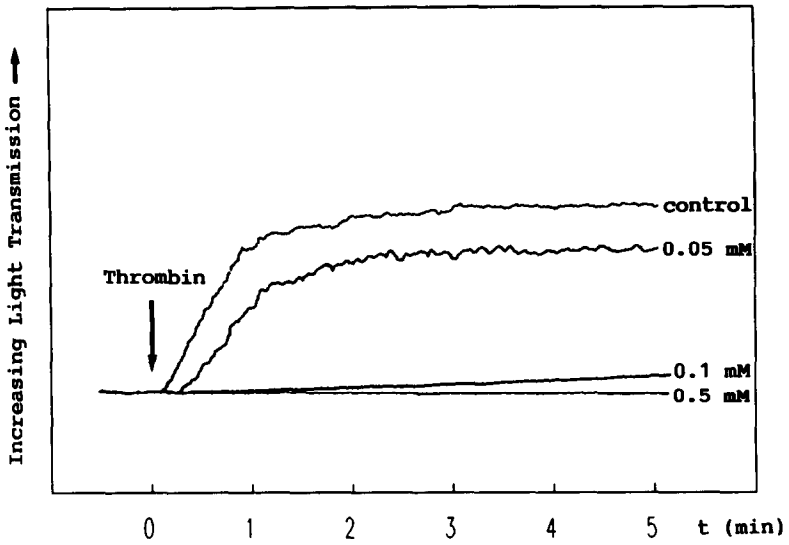

Fig. 6. Effect of 8-pCPT-cGMP on platelet aggregation. Washed human platelets were incubated for 10 min in the absence (control) or presence of 0.5, 0.1 and 0.05 mM 8-pCPT-cGMP before 0.2 U/mL thrombin was added (indicated by the arrow). Platelet aggregation was monitored by the increase in light transmission.

diesterases, platelet membranes and intact human platelets demonstrate that 8-pCPT-cGMP is a cyclic nucleotide analog useful for intact cell studies in which a selective activation of the cGMP-PK without major effects on the cAMP-PK and cGMP-regulated phosphodiesterases is desired. These data are complementary to our recent studies with Sp-5,6-dichloro-1-β-ribofuranosylbenzimidazole-3′, S′-monophosphorothioate which was characterized as a potent and selective activator of the cAMP-PK in intact human platelets [5]. We interpret our present results with 8-pCPT-cGMP as further evidence that activation of the cGMP-PK causes inhibition of aggregation of human platelets and that the effects of cGMP-elevating nitrovasodilators in human platelets are mediated by cGMP and the cGMP-PK. It should be emphasized that our results do not rule out an additional important regulatory role of cGMP-regulated phosphodiesterase in human platelets under physiological conditions. When both cAMP- and cGMP-elevating platelet inhibitors were present at low concentrations, cGMP-elevating agents potentiated the effects of prostaglandins on cAMP-levels in rabbit platelets [17, 18], an effect confirmed and extended by us with respect to the analysis of platelet cAMP levels and cAMP-PK-mediated protein phosphorylation in human platelets (data not shown). It is possible that 8-pCPT-cGMP may be an effective regulator of cGMP-gated cation channels which have a cGMP-binding site with similar specificity to those on the cGMP-PK [9]. However, the affinity of these cation channels for cGMP (and presumably for cGMP analogs) is much lower than that of those on the cGMP-PK [9]. With respect to human platelets, there is no evidence that these cells contain cGMP-gated ion channels. The activation of human platelets is strongly inhibited by cGMP-elevating nitrovasodilators such as SNP, endothelium derived relaxing factor and other NO-generating agents [6, 36–38]. Additional recent data show that NO-generating agents and 8-pCPT-cGMP also inhibit Ca^{2+}-discharge from intracellular stores in intact human platelets [35]. Our present data indicate that the inhibitory effects of cGMP-elevating nitrovasodilators and 8-pCPT-cGMP on the activation of human platelets are mediated by the cGMP-PK.

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